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ABC-transporter blockage mediated by xanthotoxin and bergapten is the major pathway for chemosensitization of multidrug-resistant cancer cells

Seyed Abbas Mirzaei[a], Neda Gholamian Dehkordi[a], Mahsa Ghamghami[b], Amir Hossein Amiri[a], Elaheh Dalir Abdolahinia[b], Fatemeh Elahian[c,*]

[a] Cancer Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran
[b] Department of Pharmaceutical Biotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Iran
[c] Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

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ABSTRACT

Furanocoumarins derived from herbal and citrus extracts can act as antibacterial, antioxidant, immunomodulator, apoptotic, and selective anticancer agents, prompting a biological investigation to determine and predict their clinical therapeutic significance. Here, the cell cytotoxic effects of bergapten and xanthotoxin were analyzed alone and in combination with standard chemotherapeutics on three multidrug resistant cells and their nonresistant parental counterparts. The furanocoumarins modulatory effects on MDR1, BCRP, and MRP pump expression and function were investigated. Although quantitative real time PCR demonstrated that the MDR transcript level changes in a time dependent manner, flow cytometric analyses using fluorescent-labeled antibodies have indicated that bergapten and xanthotoxin had no significant effect on the protein levels. FACS analyses indicated that these prominent anticancer agents significantly blocked MDR1, BCRP, and MRP transporter function. Maximum furanocoumarin-mediated pump activity blockage in the MDR-resistant cells was quantified as 87% of normal and consequently, chemotherapeutic accumulation increased up to 2.7-fold and cytotoxicity tension increased 104-fold. MDR1 efflux kinetics also revealed that the maximum velocity and the pump affinity to daunorubicin were uncompetitively decreased. We conclude that bergapten and xanthotoxin are cytotoxic agents capable of preventing daunorubicin, mitoxantrone, and cisplatin binding to ABC-transporters and subsequently inhibiting their efflux out of cells and they may be a potential combination therapy for malignant cancers.

1. Introduction

Multidrug resistance (MDR) is one of the major initial obstacles to successfully treating human malignancies and presents with concurrent acquired/innate insensitivity to a group of chemotherapeutic agents (Kondratov et al., 2001). ATP-binding cassette (ABC) transporters belong to a large protein superfamily that mediates unidirectional efflux of xenobiotics outside of cells. Current studies have shown that ABC-transporters play important roles both in cellular protection from various cytotoxic agents and the occurrence of multidrug resistance in a wide spectrum of human malignancies (Szakacs et al., 2006). MDR proteins rapidly pump anticancer drugs out of the MDR-tumor cells, decrease the intracellular concentration of chemotherapeutics below their therapeutic thresholds, and often lead to insufficient treatment (Fletcher et al., 2010). Multidrug resistance associated with BCRP, MRP, and MDR1 over-expression that extrude mitoxantrone, cisplatin, and daunorubicin are among the most prevalent occurrence of drug resistance in many cancers (Gradhand and Kim, 2008).

One of the best approaches for overcoming multidrug resistance is the use of biochemical compounds, known as MDR modulators or chemosensitizers, that decrease MDR pump function and/or expression in tumor cells (Szakacs et al., 2006). Although various chemosensitizers with different toxicities and affinities for ABC-transporters have been developed, optimal and satisfactory results have not yet been obtained. Alternatively, high therapeutic concentrations of these modulators increase vital organ toxicity (Riveiro et al., 2010; Tan et al., 2013). Thus, it is necessary to investigate other compounds with low cytotoxicity at doses required for MDR pump inhibition. Recently, it has been shown that natural products, such as fruits and vegetables, contain phytochemicals with potential anticarcinogenic properties and many studies have been directed at recognizing new compounds with improved characteristics to circumvent or even work against the various models of multidrug resistant cancers (Liu, 2003; Colabufo et al., 2010).

Coumarins belong to the benzopyrone families and are active

* Corresponding author.
E-mail address: elahian@skums.ac.ir (F. Elahian).

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components isolated from citrus fruits and vegetables. Over the last century, coumarin and its derivatives were biologically recognized as anti-coagulant, anti-microbial, antioxidant, anti-viral, anti-inflammatory, HIV-1 integrase inhibitor, enzyme inhibitor, erythroid differentiation inducer, and immunomodulator agents, supported by different clinical studies (Riveiro et al., 2010; Thakur et al., 2015). Nevertheless, this family has also exhibited promising chemotherapeutic activities at different stages of cancer through various mechanisms, such as angiogenesis inhibition, antimitotic activity, apoptosis initiation, aromatase inhibition, autophagy induction, carbonic anhydrase inhibition, cell cycle arrest, kinase inhibition, estrogen receptor modulation, sulfatase inhibition, and topoisomerase blockade. Due to the former potential applications, extensive efforts have been made to discover and synthesize coumarin derivatives with improved antineoplastic activities. Quantitative structure–activity relationship (QSAR) analyses revealed that different moieties in the coumarin nucleus strongly influence the biological activity of the resulting derivatives. In this regard, several reports have demonstrated that furan scaffolds containing coumarin structures (furanocoumarins) have a significant role in antitumor properties even more than doxorubicin (Lacy and O'Kennedy, 2004; Lipeeva et al., 2015).

There is accumulating evidence that furanocoumarins effectively prevent the proliferation of human multidrug resistant tumor cells (Lacy and O'Kennedy, 2004). Although some synergistic cytotoxicity has been observed between some furanocoumarins and common chemotherapeutic agents, little molecular information is available about their effects on ABC-transporters transcription, translation, or protein interaction in the multidrug resistant cancers. Therefore, this study was designed to obtain more information on the modulatory effects of two furanocoumarin derivatives, bergapten and xanthotoxin, on ABC-transporter expression and function in human cancer cell lines. This study offers new perspectives on the development of novel compounds in the management of multidrug resistant carcinomas.

2. Materials and methods

2.1. Chemicals and media

Bergapten, chemotherapeutic agents (cisplatin, daunorubicin, and mitoxantrone), indomethacin, novobiocin, verapamil, and xanthotoxin were purchased from Sigma-Aldrich (Sigma-Aldrich, Deisenhofen, Germany). RPMI-1640 medium, trypsin, fetal bovine serum (FBS), penicillin and streptomycin were acquired from Gibco (Grand Island, NY, USA). BCRP, MDR1, and MRP2 primary antibodies (mouse monoclonal IgG), goat anti-mouse secondary IgG1 and IgG2a conjugated with FITC, and the appropriate isotype controls were obtained from Abcam (Cambridge, USA). All other chemicals and solvents were of analytical grade and were provided by Merck (Darmstadt, Germany).

2.2. Cell lines, cell culture, and proliferation assay

A2780RCIS (MRP2 overexpressing human epithelial ovarian cancer cell line), EPG85.257RDB (MDR1 overexpressing human gastric adenocarcinoma cell line), MCF7MX (BCRP overexpressing human epithelial breast cancer cell line), and their parental lines were generously provided by Professor Herman Lage (University Medicine Berlin, Charite Campus Mitte, Berlin, Deutschland). Parental A2780, EPG85.257, and MCF7 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified CO_2 incubator at 37 °C. The media for the RCIS, RDB and MX resistant cell lines was also supplemented with 33.21 μM cisplatin, 4.74 μM daunorubicin, or 100 nM mitoxantrone, respectively (Lage et al., 2010; Elahian et al., 2013). Cellular proliferation was determined according to previously published methods; briefly, 1000 cells were seeded into each well of 96-well plates. The growth rate was evaluated during a 7-day incubation using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mansouri et al., 2014). All experiments were performed three independent times in triplicate, and the mean value ± SE was reported.

2.3. Cytotoxicity analyses and cytotoxicity synergisms

Briefly, the cells were seeded at a density of 1000 cells per well in tissue culture plates and incubated at 37 °C for 24 h. After, the cells were treated with various serial dilutions (0–100 μM) of bergapten, cisplatin, daunorubicin, mitoxantrone, or xanthotoxin. Cell viability was evaluated after a 5-day incubation of the treated cells using an MTT assay in the TECAN microplate reader (Infinite®-M200; Grödig, Austria) at 570 nm. IC_{10} and IC_{50} values were defined as the drug concentration that reduced the surviving fraction of cells in each well by 10 and 50% compared to the untreated cells, respectively. IC_{50} and IC_{10} were calculated from the best regression plot of the percentage viability versus any drug concentration. Synergistic cytotoxicity was evaluated by the combined treatment of the cells with various concentrations of the cisplatin, daunorubicin, or mitoxantrone in the presence of constant IC_{10} values of bergapten or xanthotoxin. SIC_{50} (synergistic IC_{50}) values were defined as the chemotherapeutic drug concentration that reduced the surviving fraction of cells in each well by 50% compared to the untreated cells. The SIC_{50} was determined from the equation of the best-fitting regression line plotted from the relative cell viability versus chemotherapeutic drug concentrations. All experiments were performed three independent times, and the mean values ± SE were reported (Elahian et al., 2013; Mansouri et al., 2014).

2.4. Relative quantification of the gene expression

Cells were seeded into 6-well plates at a density of 3×10^5 cells/well for 24 h. The exponentially growing cells were treated with cell-specific IC_{10} values of bergapten or xanthotoxin for 24, 48, and 72 h. Approximately 1×10^6 cells were then harvested for total RNA isolation using a highly pure RNA isolation kit (Roche Applied Science, Mannheim, Germany). One microgram of total RNA was used for reverse transcription with the first strand cDNA synthesis kit containing

Table 1
IC_{10} and IC_{50} values of furanocoumarins and standard chemotherapeutics for the normal and corresponding resistant cells, respectively.

Cell lines	Bergapten IC_{10} ± SE[a]	Xanthotoxin IC_{10} ± SE	Daunorubicin IC_{50} ± SE	Mitoxantrone IC_{50} ± SE	Cisplatin IC_{50} ± SE
EPG85.257	100 ± 10.17	120 ± 9.70	65.67 ± 1.94	ND	ND
EPG85.257RDB	40.29 ± 0.30	21,220 ± 61.51	9063 ± 136.23	ND	ND
MCF7	960 ± 92.22	990 ± 30.88	ND	620 ± 23.12	ND
MCF7MX	640 ± 71.90	1.10 ± 0.91	ND	6460 ± 101.77	ND
A2780	4260 ± 131.44	130 ± 11.75	218.54 ± 11.34	ND	1830 ± 185.66
A2780RCIS	80.34 ± 13.87	3521 ± 48.37	210.12 ± 29.78	ND	45,892 ± 1191

Data represent the mean ± standard error of three individual experiments.
ND, not defined.
[a] Drug concentration (nM) required for 10% or 50% inhibition of cell growth after 5 days of drug exposure.

random hexamer (Qiagen, Germany). Quantitative real-time analyses were performed using the 2 × QuantiTect SYBR Green PCR kit (Qiagen, Germany) in a total volume of 20 µl. The following oligonucleotide primer pairs were used: BCRP (sense 5′–TAT CAA TGG GAT CAT GAA ACC TGG-3′; antisense 5′–GCG GTG CTC CAT TTA TCA GAA C-3′; annealing 62 °C; NCBI accession: NM_004827.2), MDR1 (sense 5′–CAG CTA TTC GAA GAG TGG GC-3′; antisense 5′–CCT GAC TCA CCA CAC CAA TG-3′; annealing 56 °C; NCBI accession: NM_000927.4), MRP1 (sense 5′ –AGA GAC AGC TCA GCA GCT CCT-3′; antisense 5′ –GCC TTG TCA GCC TCC ATC AG-3′; optimal annealing 59 °C; NCBI accession: NM_004996.3) MRP2 (sense 5′–CTA CTC CAT CAA TGA TAA TCT GAC C-3′; antisense 5′–AGG ATG ACA TCA GAA ATA GAG ACC-3′; optimal annealing 59 °C; NCBI accession: NM_000392.3), and β-actin (sense 5′–TCA TGA AGT GTG ACG TGG ACA TC- 3′; antisense 5′–CAG GAG GAG CAA TGA TCT TGA TCT- 3′; annealing 60 °C; NCBI accession: NM_001101.3). Amplification was performed using the following parameters: pre-denaturation at 95 °C for 10 min; 40 cycles of 15 s at 95 °C, 20 s at annealing temperature and 20 s at 72 °C. Primer efficiency was determined prior to quantification. All experiments were run three independent times in triplicate using the Rotor-Gene Q instrument (Qiagen, Hilden, Germany) and normalized to β-actin expression in the respective samples. Relative fold changes in the expression pattern were quantified according to the Pfaffl method (Elahian et al., 2009).

2.5. Relative quantification of protein levels

MDR resistant lines and the parental counterparts were seeded at a density of 5×10^5 cells/well in 6-well tissue culture plates and incubated at 37 °C for 24 h. The cells were then treated with IC_{10} concentration values of bergapten or xanthotoxin for 24, 48, and 72 h, followed by trypsinization and washing twice with ice cold PBS. Next, the cells were fixed and permeabilized for 10 min in 10% v/v room temperature formaldehyde and 10 min in 90% v/v ice cold methanol. Subsequently, the cells were blocked for 1 h with 10% (w/v) bovine serum albumin (BSA) at room temperature to reduce nonspecific primary antibody binding. The cells were incubated for 1 h with a mouse monoclonal antibody directed against either human BCRP, MDR1, or MRP2 diluted (1:100) in PBS supplemented with 2% bovine serum albumin and 0.01% tween-20 at 4 °C. Then, the cells were treated with the corresponding FITC-conjugated goat anti-mouse immunoglobulin (1:100) in the same supplemented PBS buffer for 20 min on ice in the dark. Washing between all steps was performed with PBS at room temperature with mixing, unless otherwise specified. The MDR protein levels were determined using a BD Biosciences FACSCalibur™ flow cytometer. Cell clumps and cellular debris were excluded from the analyses using forward/side scatter gating. Cells were excited with a standard argon laser at 488 nm, and the emission was recorded via a 530/30 nm band-pass filter (FL1). Appropriate autofluorescence, isotype, and secondary antibody controls were also considered to minimize the non-specific background signals caused by the unsatisfactory reaction conditions. Flow cytometry data were processed and analyzed using WinMDI version 2.8. All assays were performed in at least three independent experiments, and relative protein levels were considered as the mean fluorescence intensities of treated cells compared to the untreated controls (Elahian et al., 2009).

2.6. Quantitative kinetics of transporter blockade

ABC-transporter functionality in the presence and absence of selected furanocoumarins was quantified by flow cytometry. Briefly, cells were seeded at 5×10^5/well in 6-well plates and were treated with IC_{10} concentrations of bergapten or xanthotoxin for 24, 48 and 72 h. Following trypsinization, the cells were resuspended and divided equally in medium containing MDR fluorescent substrates (daunorubicin for MDR1 and MRP pumps and mitoxantrone for BCRP) alone or in combination with the corresponding specific pump inhibitor

Table 2
SIC_{50} values of standard chemotherapeutics in the presence of bergapten or xanthotoxin for the normal and corresponding resistant cells.

Cell lines	Daunorubicin SIC_{50} ± SE in the presence of Bergapten	Daunorubicin SIC_{50} ± SE in the presence of Xanthotoxin	Mitoxantrone SIC_{50} ± SE in the presence of Xanthotoxin	Mitoxantrone SIC_{50} ± SE in the presence of Bergapten	Cisplatin SIC_{50} ± SE in the presence of Xanthotoxin	Cisplatin SIC_{50} ± SE in the presence of Bergapten
EPG85.257	15.50 ± 0.14	4.73 ± 0.13	ND	ND	ND	ND
EPG85.257RDB	88.75 ± 0.09	6435.45 ± 25.1-2	ND	ND	ND	ND
MCF7	ND	ND	521 ± 10.02	190 ± 20.89	ND	ND
MCF7MX	ND	ND	2891 ± 55.18	2788 ± 110.8	ND	ND
A2780	32.44 ± 3.45	31.37 ± 8.94	ND	ND	950 ± 142.11	1390 ± 196.-22
A2780RCIS	110.93 ± 6.10	201.77 ± 10.09	ND	ND	38,861 ± 138	31,055 ± 533

Data represent the mean ± standard error of three individual experiments.
ND, not defined.
Chemotherapeutic concentration (nM) required for 50% inhibition of cell growth after 5 days of drug exposure.

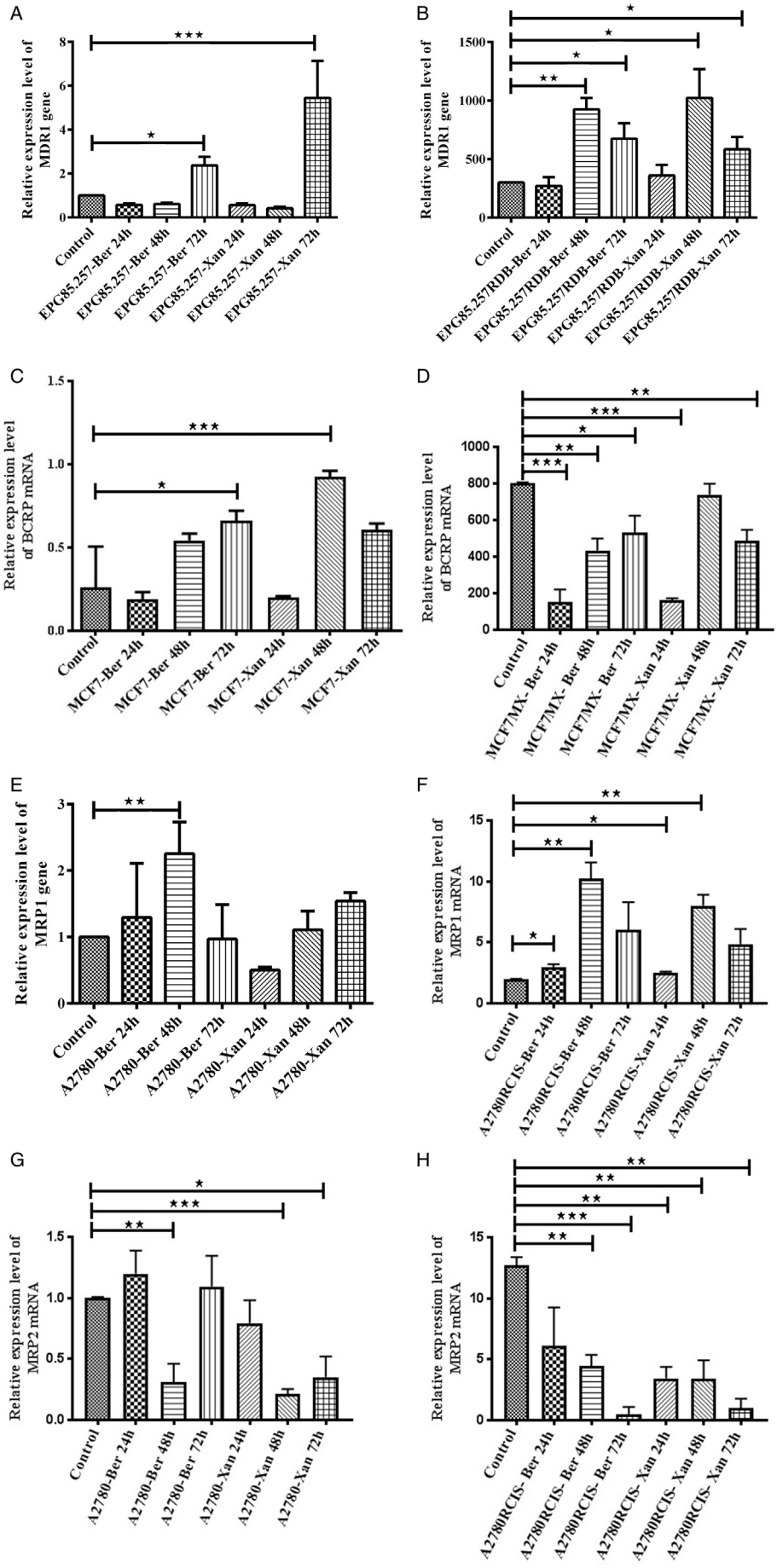

Fig. 1. Relative quantifications of MRP1, MDR1, and BCRP transcripts in EPG85.257, MCF7, A2780 and their resistant counterparts. Cells were incubated with IC_{10} concentrations of any furanocoumarins for 24 h, 48 h, and 72 h. Fully quantitative measurement of mRNA transcripts was conducted using the Pfaffl method, and β-actin was used as a preferred internal control. The results are presented as mean ± SE of three independent experiments each in triplicate compared with untreated samples. Symbols (***), (**), and (*) represent the mean differences between parental and resistant cell transcriptions as $P < 0.001$, $P < 0.01$, and $P < 0.05$ using one-way ANOVA, respectively.

(verapamil for MDR1, indomethacin for MRP and novobiocin for BCRP). After a 30 min incubation period at 37 °C, the cells were harvested at 800 × *g* and washed twice with ice cold PBS. Then, the cell suspensions were equally divided again into two fractions and one portion was placed on ice in the dark for FACS analyses of the accumulation kinetics. The remaining cell portions were treated with complete medium supplemented with (inhibitor treated cells in the accumulation steps) or without (inhibitor free treated cells in the accumulation steps) their specific inhibitors. The cells were then incubated at 37 °C for 1 h, washed twice with ice cold PBS, and maintained in the dark for FACS analyses for efflux kinetic experiments. IC_{10} values of furanocoumarins were present in all steps of kinetic assays. Forward/side scatter was used to excluded cell clumps and cellular debris. Cells were excited with a standard argon laser at 488 nm and the emission was recorded via a 585/42 nm band-pass filter (FL2, in the cases of daunorubicin used as the fluorescent substrate) and 670 nm long-pass filter (FL3, for mitoxantrone treated cells). All experiments were performed at least three independent times, and the cells receiving no furanocoumarin treatments were used as negative controls. The following equations represent the mathematical models of the procedure. MFI, modulator, sample, and control are mean fluorescent intensity, inhibitor (verapamil for MDR1, indomethacin for MRP, or novobiocin for BCRP), furanocoumarin treated cells, and untreated cells, respectively (Elahian et al., 2013; Zhang et al., 2014).

$$\Delta\text{Efflux} = ((\text{MFI}_{(\text{with modulator sample})} - \text{MFI}_{(\text{without modulator sample})}) \div (\text{MFI}_{(\text{with modulator control})} - \text{MFI}_{(\text{without modulator control})})) \times 100\%.$$

$$\text{Drug accumulation} = (\text{MFI}_{(\text{without modulator sample})} \div \text{MFI}_{(\text{without modulator control})}) \times 100\%$$

2.7. Statistical analyses

All experiments were performed at least three independent times. Quantitative data was statistically analyzed using SPSS-22 software. Differences between groups were determined by one-way ANOVA with post hoc Tukey's HSD test, and *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Cell proliferation and cytotoxicity assays

Statistical analyses revealed that the maximum specific growth rate of the parent lines and their resistant counterparts during the first four days of exponential growth is almost equal ($P > 0.05$); the parent cell lines continue logarithmic growth but the resistant cells return to a stationary phase, which led to a lower biomass (Supplementary Table S1). Cytotoxicity values of each chemotherapeutic agent and furanocoumarin were calculated from a dose-response curve that was fitted to the resulting data. All of the data were calculated after 5 days of exposure using MTT cell viability assays (Supplementary Fig. S1), and the results of three independent experiments were reported as the mean ± SE (Table 1). Synergistic cytotoxicity effects of bergapten and xanthotoxin with any of the chemotherapeutic agents were also calculated from combination treatments (Table 2, Supplementary Fig. S1). The data show that both furanocoumarins synergistically increased the cytotoxicity of cisplatin, daunorubicin, or mitoxantrone in the resistant cell lines compared to the single standard chemotherapy ($P < 0.001$). This phenomenon may be due to the effects of xanthotoxin and ber-

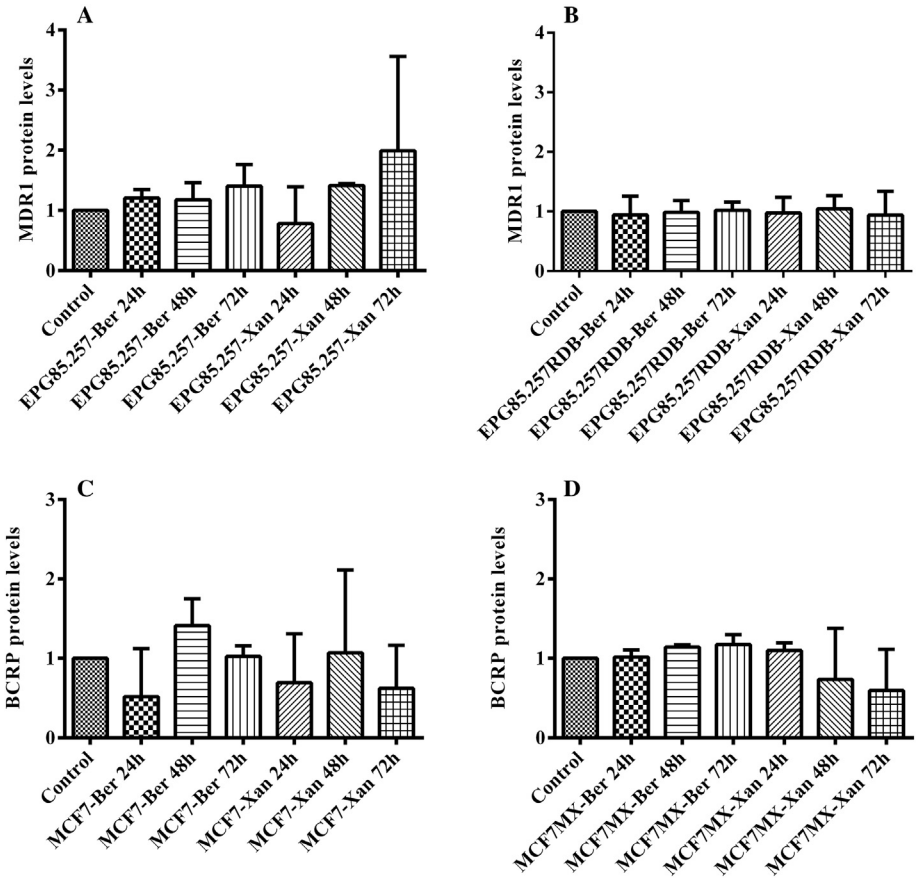

Fig. 2. Quantitative FACS analyses of MDR proteins. MDR1 and BCRP protein levels of EPG85.257, MCF7, and their resistant counterparts were quantified in the presence of bergapten and xanthotoxin using flow cytometry. Fold changes were calculated relative to the untreated conditions for any cells, and the average was calculated from three independent experiments and graphed as mean ± SD.

gapten on the MDR down-regulation and/or physical inhibition of the ABC efflux pump activity.

3.2. MDR pump transcript and protein quantification

The relative expression of the *BCRP, MDR1*, and *MRP2* genes in the presence and absence of IC_{10} doses of bergapten or xanthotoxin was assessed using real time PCR. Primer sequences and amplification conditions were optimized to obtain the best amplification efficiency ($E \geq 1.9$ and R-squared ≥ 0.95) according to the standard Pfaffl method. BCRP, MDR1, and MRP2 mRNA expression in MCF7MX, EPG85257RDB, and A2780RCIS were measured at approximately 800-, 310-, and 13-fold more than the transcript contents of their parent counterparts, respectively. Parent cells showed barely perceptible MDR transcript level changes after any treatments, because they naturally produce low levels of any MDR pumps and are classified as sensitive cells that lack efflux function. MDR1 and MRP1 transcripts represented few folds of up-regulation time-dependently and BCRP and MRP2 experienced mild down-regulation in their corresponding resistant cells after any furanocoumarin treatments. Maximum up- and down- regulations were recorded near 3-fold from MDR1 and BCRP transcripts after 48 h and 24 h of both treatments, respectively. Generally MRP1 transcript was less affected after any treatments (Fig. 1). Fig. 2 presents data acquisition from the flow cytometer and reveals no ABC-transporter protein level changes during the first three days after any treatment with bergapten or xanthotoxin ($P > 0.05$).

3.3. Furanocoumarins effects on MDR transporter activity

Bergapten- or xanthotoxin-treated parental A2780, EPG85.257, and MCF7 cells (without any active MDR pumps) did not have a considerable stimulatory effect on daunorubicin (for A2780 and EPG cell lines) or mitoxantrone (for MCF7 cells) accumulation and did not preserve significant effects on MDR pump efflux activity over 72 h ($P > 0.05$) (Supplementary Fig. S2).

Bergapten and xanthotoxin enhanced daunorubicin accumulation in A2780RCIS and EPG85.257RDB during the first 72 h of exposure ($P < 0.01$). Dose-response curves indicate time-dependent involvement of daunorubicin accumulation in both cells (Supplementary Fig. S3). Maximum daunorubicin accumulation was observed after 24 h and 48 h of drug exposure in A2780RCIS and EPG85.257RDB, respectively ($P < 0.001$). Both treatments resulted in a dynamic reduction of MDR1 pump activity in EPG85.257RDB cell lines (Supplementary Fig. S3) during the first three days ($P < 0.001$). Although MRP-dependent daunorubicin efflux returned to the control rate after 72 h exposure to xanthotoxin in A2780RCIS cells, drug accumulation increased. Therefore, the inhibition of oxidoreductase pathways for drug metabolism is the major reason for this phenomenon (Fig. 3 and Supplementary Fig. S3).

Fluorescence intensity values significantly increased ($P < 0.001$) in bergapten- or xanthotoxin-treated MCF7MX cells in a time dependent manner. Alternatively, bergapten or xanthotoxin decreased the mitoxantrone efflux in MCF7MX cells after 24 h, 48 h and 72 h ($P < 0.001$)

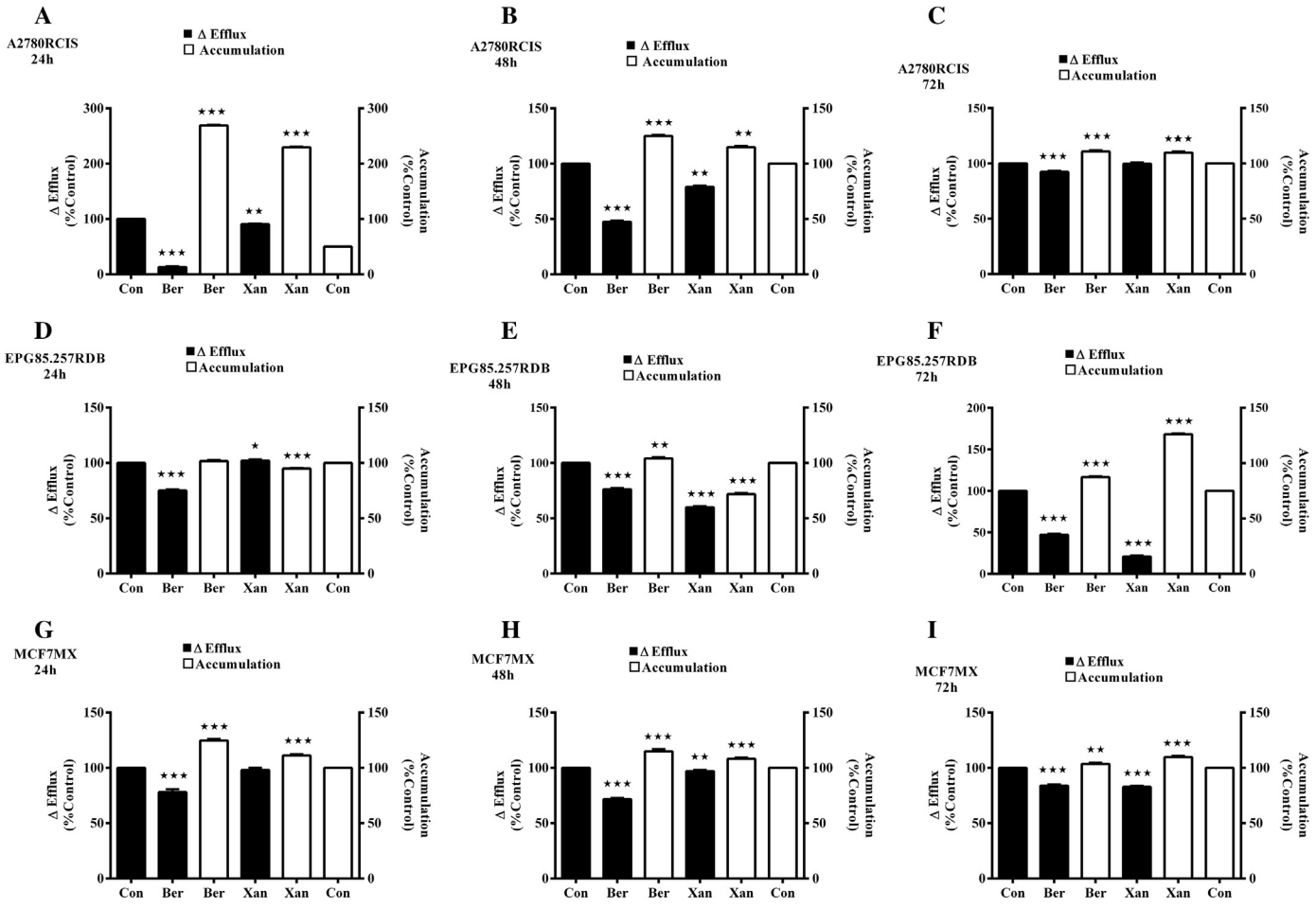

Fig. 3. Flow cytometry analyses of furanocoumarin effects on daunorubicin and mitoxantrone efflux and accumulation in MDR resistant cells. Cells were pretreated with IC_{10} concentrations of bergapten or xanthotoxin for 24 h, 48 h, and 72 h and then incubated with IC_{50} values of daunorubicin or mitoxantrone in the presence or absence of a specific inhibitor (indomethacin for MRP pump, verapamil for MDR1 pump, or novobiocin for BCRP pump). Efflux and accumulation were calculated from the mean intensity of fluorescent chemotherapeutics and presented as the mean ± SE. The symbols (***), (**), and (*) represent the mean fluorescence difference between furanocoumarin treated and untreated cells as $P < 0.001$, $P < 0.05$ and $P < 0.01$, respectively. Con, control; Ber, bergapten; Xan, xanthotoxin.

(Fig. 3 and Supplementary Fig. S4).

4. Discussion

The literature shows that many neutral and synthetic coumarins are significant cancer cell growth inhibitors in vitro and in vivo. Coumarin derivatives participate in cell death through inhibition of cellular proliferation cascades and activation of regulatory and apoptotic proteins (Jun et al., 2014). Studies have shown that a substituent on C3, C4, C7 and C8 of the coumarin core structure with amide, hydrazide, aryl and imine groups has a definite role in selective antitumor activity (Emami and Dadashpour, 2015). Therefore, furanocoumarins are more potent antioxidant, immunomodulatory, cytotoxic, and cytostatic agents and growth modulators (Piao et al., 2004; Finn et al., 2005; Pingaewa et al., 2014).

Unfortunately, overexpression of ABC-transporters encoded by multidrug resistance genes are among the major obstacles for successful cancer chemotherapy (Szakacs et al., 2006). In this regard, natural coumarins in combination with common chemotherapeutic agents provide increased drug bioavailability by interfering with drug metabolism and ABC-transporters and strong synergistic interactions against multi drug resistant cancer cells (Kimura et al., 2005; Efferth and Volm, 2017). Previously, bergaptol, bergapten and bergamottin, as famous furanocoumarins in grapefruit juice, have increased the steady-state uptake of vinblastine in the cells. The mechanisms conferring these capabilities may include direct interaction of the drugs with MDR pump expression and function (Ohnishi et al., 2000) or enhanced cytotoxicity using the elimination of oxidoreduction enzyme cascades (Elahian et al., 2014; Elahian et al., 2016). Consistent with other studies, the results described in Tables 1 and 2 confirmed that cisplatin, daunorubicin, or mitoxantrone cytotoxicity was remarkably increased in the most resistant cancer cells treated with non-toxic concentrations of xanthotoxin and bergapten.

Although some fluctuations were observed in *BCRP*, *MDR1*, and *MRP* gene expression in the parental and resistant cell lines during the examination time, the gene expressions almost returned to a steady state level after 72 h of drug exposure (Fig. 1). Alteration in the activity and expression of ABC-transporters seems to be a primary cell response, necessary for xenobiotic detoxification and adaptation processes (Fromm, 2002; Fromm, 2004). A few fold change in transcript levels (non-significant with $P > 0.05$) have also been previously reported for similar compounds (Hsieh et al., 2014); our results again demonstrated diversity in furanocoumarins effects on MDR gene expression and protein levels, which are most likely cell and even pump specific (Zhao et al., 1993; Canitrot et al., 1998; Pulaski et al., 2005).

Alternatively, bergapten and xanthotoxin enhanced intracellular daunorubicin or mitoxantrone accumulation in the resistant cancer cell lines. Since MDR protein levels were not significantly changed in the presence of the furanocoumarins, we concluded that the stimulatory effect of bergapten and xanthotoxin on cellular chemotherapeutic agent accumulation was due to their inhibitory effects on MDR1, BCRP, and MRP functions. These data are consistent with cytotoxicity; when the bergapten limited the MDR1 pump activity to 47% of untreated cells, daunorubicin accumulation increased 116% in this cell type and EPG85.257RDB sensitivity to daunorubicin toxicity increased 102-fold. A2780RCIS cells were 48-fold more sensitive to cisplatin and drug accumulation increased 269% when furanocoumarins decreased the efflux activity 13%. Upon transient exposure of MCF7MX cells to furanocoumarins, a parallel increase in mitoxantrone cytotoxicity up to 2-fold and mitoxantrone efflux reduction of approximately 71% of normal occurred (Tables 1, 2 and Fig. 3). Although in some cases the efflux activity returned to normal after 72 h of furanocoumarins treatment, the chemotherapeutic agent accumulation followed an ascending trend (Fig. 3). The mechanism conferring this enhanced bioavailability may include inhibition of intercellular oxidoreduction and degradation of daunorubicin and mitoxantrone via inhibition of CYP3A and CYP1A1/2 enzymatic activities in the catalyst of phase I and phase II drug biotransformation. Obviously, these small molecules may also inhibit cytochrome enzyme activity directly through binding to them, which may also be useful for cancer treatment (Wen et al., 2002; Kim et al., 2014).

The results suggested that bergapten and xanthotoxin act as ABC-transporter inhibitors and the presumable underlying mechanism of their inhibitory effect could be derived from their ability to interact with ABC-transporters and interfere with the efflux activity, increasing the intracellular drug accumulation (Nabekura, 2010). Consistent with our observation, previous studies have shown that cnidiadin, bergamottin, and psoralen increase the bioavailability and accumulation of MDR1 substrates by partially inhibiting transporter activity (Ohnishi et al., 2000; Honda et al., 2004). Previously, several natural compounds have been defined as MDR transporter competitive or uncompetitive blockers as they occupy the drug binding sites or block the ATP hydrolysis process in MDR pumps (Safa, 2004; Marchetti et al., 2007). MDR1 pump kinetic analyses here showed a potent interaction of the furanocoumarins with daunorubicin, accumulating the chemotherapeutic agent in the MDR cells, inhibiting growth, and causing cell death. Kinetic analyses revealed that the maximum velocity of efflux (V_{max}) and the MDR1 affinity to daunorubicin (Km) were significantly decreased in the EPG85.257RDB cells ($P < 0.001$) treated with bergapten ($V_{max} = 23.68 \pm 0.58$ μM/min and $Km = 2.24 \pm 0.64$ μM) or xanthotoxin ($V_{max} = 12.94 \pm 1.14$ μM/min and $Km = 1.60 \pm 0.91$ μM). Even the normal V_{max} and Km for the MDR1 pump were calculated to be 66.24 ± 5.90 μM/min and 6.73 ± 0.70 μM, respectively, thus, representing uncompetitive inhibition on MDR1 (Erzengin et al., 2012).

Finally, some controversies in the accumulation and efflux results in furanocoumarin-treated parental cells are mainly due to the absence of a sophisticated efflux pump in these cells and probable activation of parser compounds in the cells, activation of degradation pathways, the existence of a variety of nonspecific pumps on parental cells that are involved in xenobiotic transportation out/in, or the expression of some members of organ anion dependent families that can reduce the intracellular concentration of chemotherapeutic agents (Albertus and Laine, 2001; Deeley et al., 2006).

5. Conclusion

Our results convincingly demonstrated that selected furanocoumarins are capable of uncompetitively impeding the binding and efflux of chemotherapeutic agents by ABC-transporters. Since one of the main causes of failure in chemotherapy is related to inherent or acquired drug resistance due to over expression of efflux pumps by tumor cells, the potency of bergapten and xanthotoxin in reversing drug efflux would make these components potential candidates in clinical studies in future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2017.10.018.

Conflict of interest

We certify that there is no financial conflict of interest involving any organization.

Author contributions

F. Elahian coordinated the study, designed FACS experiments, performed the statistical analyses, and revised the final manuscript. S.A. Mirzaei, N. Gholamian, and E. Dalir designed and performed real time PCR studies, protein quantification, and efflux kinetics, wrote the corresponding portions of the manuscript, and participated in intellectual discussions of the data and manuscript writing. M. Ghamghami and A. H. Amiri performed cytotoxicity and FACS experiments as parts of their theses in PharmD degree and master degree in medical biotechnology, respectively.

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